THE MINERAL REQUIREMENTS FOR SPORULATION*

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Although scores of papers have been published on various aspects of spore formation, relatively few have been concerned directly with the role of minerals in sporogenesis. Salt effects on sporulation were reported by Behring as early as 1889, and by Schreiber in 1896. However, their real significance in the production of spore was not recognized until many years later. Thus, Cook, in his comprehensive review "Bacterial Spores" (1932), concluded "it does not appear that salts exert a direct influence on sporulation." The inability of early workers to devise chemically defined media that would produce spores in quantity, contributed to the slow development of knowledge in this field. Cook (1931) and Tarr (1932) were the first to overcome this difficulty, the latter, by showing that good sporulation by several anaerobic species could be obtained by cultivation in a mineral salts medium containing low concentrations of secondary ammonium phosphate and sucrose. Roberts (1934) obtained 60-70% sporulation of Bacillus subtilis with suitable minerals supplemented with asparagine and levulose. Leifson (1931) studied the effects of inorganic salts on the sporulation of Clostridium botulinum in 1% peptone. The basal medium, which by itself produced no spores, was supplemented with a variety of mineral salts used alone or in combination. Sporulation occurred only when the supplement contained NH_4^+ and PO_4^- and to some extent SO_4^- ions. The Ca^{++} ion stimulated sporulation when added to NH_4^+ and PO_4^- ions.

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Further evidence that peptone is deficient in minerals for sporulation was supplied by Fabian and Bryan (1933), who observed greatly increased sporulation of four mesophilic aerobes when the peptone solution was suitably fortified with cations of the univalent chloride salts; di- and trivalent ions were without effect. Meat infusion trypton glucose broth was shown by Knaysi (1945), to be deficient in Mg^{++} for the sporulation of Bacillus mycoides. The work of Foster and Heligman (1949) indicated that not only peptone but most complex organic media, when used in the fluid state, do not provide an adequate supply of minerals for the sporulation of Bacillus cereus; the chief limiting factor in asporogenic media was shown to be an insufficiency of K^{+}. The specificity of Mn^{++} for the sporulation of B. subtilis was first shown by Charney and his associates (1951). In both chemically defined and in complex organic media, sporulation was negligible without added Mn^{++}, although this supplement was not required for full vegetative growth.

These findings were confirmed and extended by Curran and Evans (1954), who also observed that iron, when used in relatively large amounts, replaced Mn^{++} in Mn-deficient media. Weinberg (1955) concurred in these findings but suggested that the sporogenic activity of the added $Fe^{++(+)}$ could be largely attributed to appreciable quantities of Mn^{++} contained as impurity in CP grade Fe compounds. In agreement with Weinberg, we have since found that a sample of $FeCl_3$ which in nutrient broth exhibited characteristic sporogenic activity for B. subtilis at 200 μg/ml, when purified by repeated extraction with HCl and ether became sporogenically inactive in the same medium at levels of iron in excess of 200 μg/ml.

The spore-inducing property of the medium containing purified iron could be restored by small additions of Mn^{++}.

The careful studies of Grelet (1951, 1952-a, 1952-b) have contributed valuable information concerning the minerals required for sporulation by Bacillus megatherium. With shake cultures in a glucose mineral salts medium, the effect on sporulation was determined for a number of mineral constituents, each constituent of the medium was decreased independently, and its approximate limiting concentration for sporulation determined. Depletion of glucose and nitrate in the control medium was followed by rapid and almost complete sporulation; similarly, sporulation remained at a high level with progressive reductions in the NO_3^+, SO_4^{-}, Cl^-, Na^+, $Fe^{++(+)}$, and Zn^{++}. In contrast, depletion of carbon and nitrogen occurring in conjunction with a deficiency of K^+, Mg^{++}, or Mn^{++}, prevented most of the bacilli from sporulating, indicating the essentiality of these minerals for sporulation. A deficiency of Ca^{++} and PO_4^{--} reduced the number of spores, and omission of Ca^{++} from the formula, although without effect on growth, greatly hindered sporogenesis, and yielded spores slightly refractile and weakly thermoresistant. Omission of Ca^{++}, but with Mn^{++} at a concentration equal to that of Ca^{++} in the control medium, yielded spores about equal in number to that of the control but with only 1/10 as many thermoresistant cells. When both Ca^{++} and Zn^{++} were omitted, sporulation did not occur; with omission of Zn^{++}, some Ca^{++} was necessary for sporulation.

A chemically defined medium for Bacillus anthracis was described by Brewer et al. (1946), which produced yields in excess of one billion spores/ml. The medium contained a mixture of 18 amino acids, glucose, $NaHCO_3$, glutamine, nucleic acid components, mineral salts, and vitamins. Employing a more dilute version of this medium including the nucleic acid components, Brewer studied the effects of Ca^{++}, $Fe^{++(+)}$, Mg^{++}, and Mn^{++} ions on sporulation. Each ion was tested individually by varying its concentration, while the other ions were present in approximately optimal concentration, as determined by preliminary tests. In the absence of Mg^{++}, practically no growth occurred, 1.6 μg/ml and higher of Mg^{++} induced good growth and sporulation. A good yield of spores was obtained in the absence of added calcium; however, addition of 20 μg/ml of Ca^{++} increased the spore yield about 4.5 times. Inclusion of $Fe^{++(+)}$ in the formula was not critical for sporulation, although the addition of 0.7 μg/ml increased the spore yield about 2.5 times. Added Mn^{++} was not essential to sporulation, but as recognized by the authors, enough was probably present as an impurity to provide sporulation requirements of the organism. Replacement of the K phosphates with Na phosphates decreased spore yield about 1/3. The addition of Cu^{++}, Zn^{++}, Cd^{++}, and Co^{++} at levels of 0.1-1.0 μg/ml of metallic ions, was without effect upon sporulation.

The influence of mineral salts on sporulation is reflected in the results of experiments involving the use of a nutrient medium both in fluid form and solidified with agar. Thus Roberts and Baldwin (1937) observed that agar in amounts as small as 0.06% when added to 1% peptone

solution definitely increased spore production of B. subtilis, and subsequently (1942) that the percent sporulation in vigorously aerated peptone broth was considerably less than in unaerated medium made slightly viscous with agar. Since sporulation of B. subtilis is more rapid and attains materially higher levels in shake than in stationary cultures, these results must be attributed in part at least to minerals supplied by the agar--the latter contributes relatively large amounts of Mg^{++}, K^{+}, and Ca^{++} and biologically significant amounts of Mn^{++} and $Fe^{++(+)}$.

The mineral requirements of thermophiles in relation to sporulation have received very little attention. Ward (1947) observed that sporulation of Bacillus thermoacidurans in proteose-peptone agar was increased by the addition of Li^{++}, Mg^{++}, Ca^{++}, $Fe^{++(+)}$, Zn^{++}, or Mn^{++}.

The addition of NO_3^- or NO_2^- to nutrient agar has been reported to increase the sporulation of Bacillus stearothermophilus (Dahl 1955). Schmidt's studies on the same organism, limited to NaCl and $CaCl_2$, indicated no clear-cut effect--subsequently the same investigator observed that the addition of Mn^{++} to nutrient agar greatly increased sporulation of B. S B. stearothermophilus (unpublished data). Sporulation by Bacillus coagulans var. thermoacidurans in 3 peptone-containing agar media was shown to be greatly enhanced by the addition of Mn^{++}, Co^{++}, or Ni^{++} (Amaha et al. 1956).

Apart from their utility in regulation of pH, bicarbonates may promote the formation of spore by increasing CO_2 concentration as shown by Powell and Hunter (1955).

Proliferating vegetative cells, when centrifuged, washed, and shaken in distilled water, form spores, but only if the prior growth medium is

conducive to sporulation. However, *B. subtilis* derived from an asporogenous medium sporulates in distilled water when supplied with both yeast extract and salts, but not if one of these supplements is omitted (Murrell 1955), indicating that the spore-generating mechanism is not seriously impaired by limited cultivation in asporogenous media.

The known facts, briefly surveyed, indicate that mineral salts are essential for the formation of bacterial spores. Supporting this is the fact that synthetic media without added salts, do not produce spores and the well known inadequacy for spore formation of many complex organic media which is corrected by the addition of suitable minerals. The level of minerals required to satisfy the requirements for sporulation varies with the organism and with cultural conditions, including kind and concentration of nutrients, oxygen supply, temperature, pH, etc. The importance of manganese in the sporulating process is conspicuously evident. It is required by a wide variety of mesophilic and thermophilic aerobes, at concentration levels above that needed for active vegetative growth and with one apparent exception cannot be replaced by any other mineral element. Manganese has been found to broaden the temperature and pH range over which sporulation occurs (Amaha *et al.* 1956).

Specifically what function the minerals perform in the spore-forming process is unknown. It may be presumed that they exercise a catalytic role in the activation of many enzyme systems. They may thus expedite the complex enzymatic processes involving intracellular protein degradation and resynthesis postulated by Hardwick and Foster as the basic mechanism in sporogenesis. There is evidence that organic or inorganic phosphorus may

supply some of the energy used for the synthesis of spore constituents. The presence of calcium above certain minimum levels seems to be a factor in the production of thermo-stable spores; it may well be that calcium contributes to the stability of spores by forming internal bonds with low molecular weight peptides which comprise much of the spore substance.

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